Breast Cancer (2018) 25:698–705
https://doi.org/10.1007/s12282-018-0875-3

ORIGINAL ARTICLE

Clinicopathological significance of cancer stem cell markers CD44 and ALDH1 expression in breast cancer

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Received: 15 March 2018 / Accepted: 20 May 2018 / Published online: 29 May 2018

Abstract

Background CD44 and aldehyde dehydrogenase 1 (ALDH1) has been reputed to be cancer stem cell (CSC) markers in breast cancer. Yet, the clinicopathologic and prognostic significance of these markers remain unclear. In this study, we have investigated the expression of these markers and their relation with conventional clinicopathologic tumor characteristic including molecular subtype.

Methods CD44 and ALDH1 expression were investigated by immunohistochemistry in a series of 157 formalin-fixed paraffin-embedded breast cancer tissues.

Results Overall, CD44 and ALDH1 are, respectively, detected in 33% (52 of 157) and 7% (10 of 157) of breast cancer cases. We also observed that CD44 expression was associated with histological grade ($p=0.005$). For ALDH1, we found that its expression is more frequent with elderly women (> 50 years, $p=0.03$). The investigation of relationship between the stem cell phenotype and breast cancer molecular subtype, revealed that CD44 and ALDH1 expression was more frequent in basal-like tumors ($p=0.005$). Among the two cancer stem cell markers tested, ALDH1 showed a strong association with the basal marker EGFR ($p=0.05$).

Conclusions These findings suggest that CD44 and ALDH1 play a role in the clinical behavior in breast cancer and might be interesting biomarkers and therapeutic targets.

Keywords CD44 · Aldehyde dehydrogenase 1 · Cancer stem cell · Breast cancer · Basal-like

Introduction

Breast cancer is the principal cause of cancer death among women, with more than 1,500,000 cases worldwide each year [1]. Breast cancer is a complex disease with a large heterogeneity, leading to highly variable clinical behavior and response to therapy [2]. However, the mechanisms resulting in this heterogeneity in breast cancer are not well-understood [3].

One possible explanation for the tumor heterogeneity is the cancer stem cells. These cell subpopulations have the capacity to self-renew and differentiate into multiple cell types, and may contribute to drug resistance that promotes tumor recurrence or metastasis [4]. In breast cancer, cancer stem cells are principally identifiable by the expression of CD44 and ALDH1 [5].

CD44 is a class I transmembrane glycoprotein that serves as the primary receptor for hyaluronan and binds other extracellular matrix components, such as collagen, laminin, and fibronectin [6]. This protein has been shown to promote growth, invasion, and metastatic dissemination in breast cancer cells [7, 8].

ALDH1, an enzyme responsible for the oxidation of intracellular aldehydes, has been a subject of research focus in recent years [9, 10]. Previous researches reported that ALDH1 contributed to normal and tumor stem cell differentiation as well as invasion and metastasis in breast cancer [11, 12].

Based on recent information, it is evident to support the use of the CD44 cell surface marker in combination with the ALDH1 activity as an accurate method to identify cancer stem cells within breast tumors [5].

Recently, many clinical studies have reported that tumors expressing cancer stem cell markers are associated with

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aggressiveness and with tumor progression [3, 13]. However, other researches do not confirm this observation [14, 15]. This discrepancy makes it important to further investigate the expression of these markers in breast cancer to assess their pathologic and clinical significance.

In the current study, we have examined the expression of the proposed breast cancer stem cell markers CD44 and ALDH1 in a series of breast cancer. In addition, we have investigated whether the expression of these markers is associated with conventional clinicopathologic tumor features.

Patients and methods

Patients

This study includes 157 invasive ductual breast carcinomas obtained from the archives of the Department of Pathology, Farhat Hached Hospital of Sousse (Tunisia). The cases were selected based on the availability of sufficient paraffin-embedded tissue, before any treatment.

The patients' age at diagnosis ranged from 31 to 87 years, with a mean of 48.9 years and a median of 46 years. Tumors were graded according to the modified Scarff-Bloom-Richardson system. The clinical stage of the disease was determined according to the tumor-node-metastasis (TNM) classification of the International Union against Cancer (UICC). Table 1 lists clinical and pathological characteristics of the patients, including age, histological grade, tumor size; hormone receptors (estrogen (ER), progesterone (PR)) and HER2 are available for all the cases. Axillary lymph node status was available for 129 patients.

For all patients, the surgical procedure consisted of patey mastectomy in conjunction with post-operative irradiation, chemotherapy and/or hormonal therapy according to standard protocols.

Tumors were grouped according to their ER, PR, and HER2 immunohistochemical status into 4 intrinsic subtypes according to Goldhirsch et al. [16]: luminal A (ER+ and/or PR+, HER2−, low Ki67), luminal B (ER+ and/or PR+, HER2+ and/or high Ki67), HER2 overexpressing (ER−, PR−, HER2+) and triple negative (ER−, PR−, HER2−). Taking into account the expression of basal markers cytokeratin 5/6 and EGFR, we classified triple negative tumors into basal-like (CK5+/6 and/or EGFR+) and non basal-like tumors (CK5/6− and EGFR−) [16].

Immunohistochemical identification of CD44 and ALDH1

The expression of cancer stem cell markers CD44 (clone DF1485, 1:100, Leica, Newcasttle, UK) and ALDH1 (clone 400M-15, 1:100, Cell Marque, Rocklin, California, USA) was investigated by immunohistochemistry using the EnVision Flex system (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions.

Briefly, paraffin-embedded breast cancer tissues were cut at 5 µm, dried overnight at 60 °C and deparaffinized in Ottix Plus (Diapath, Martinengo, Italy). Subsequently, the sections were hydrated with Ottix Shapper (Diapath, Martinengo, Italy), and rehydrated in water.

For antigen retrieval, the sections were boiled in a water bath with citrate buffer (0.01 M, pH 6.0) for 40 min until the temperature reached 98 °C. The sections were then allowed to cool at room temperature for 20 min. Later, they were and placed in EnVision Flex Wash buffer (DakoCytomation, Glostrup, Denmark). The endogenous peroxidase activity was blocked with EnVision Flex Peroxidase-Blocking Reagent for 5 min. The sections were thoroughly washed with the Wash buffer. The samples were incubated at 4 °C overnight with the primary antibody. Subsequently, the sections were rinsed gently with Wash buffer.

Immunostaining was performed using the high sensitive polymer-based EnVision Flex /HRP system. After being rinsed in wash buffer, the sections were incubated in 3, 3 diaminobenzidine, a substrate–chromogen solution for 20 min. Finally, the slides were counterstained with Mayer hematoxylin, permanently mounted, and viewed with a standard light microscope.

Evaluation of immunostaining

In all the cases, immunostaining results were evaluated independently by two pathologists (M.T. and S.Z.). CD44 positive staining was evaluated in the cell membrane. For ALDH1, cytoplasmic staining was detected, whereas nuclear staining alone was considered nonspecific and was not included in the analysis. For the two antibodies, a case was considered positive if more than 10% of the cells exhibited immunostaining for this antigen, otherwise, it was negative [17].

Statistical analysis

Statistical analysis was carried out with the SPSS software package (version 20.0; SPSS, Chicago, IL, USA). The correlation between the patients' clinicopathologic features, CD44 and ALDH1 expressions was investigated by the Chi square test or Fisher exact test, where appropriate. A p value ≤ 0.05 was considered to indicate statistical significance.

Results

CD44 and ALDH1 expression in breast cancer

We analyzed CD44 and ALDH1 to identify the breast cancer cases with stem cell phenotype. Overall, 55 of the 157 (35%)

Table 1 Correlation between breast cancer stem cell markers and the classic clinicopathological parameters and intrinsic molecular subtypes

	Total	CD44 expression		ALDH1 expression			ALDH1/CD44 expression	
		n (%)	*p* value	*n* (%)	*p* value		*n* (%)	*p* value
Age (years)								
≤ 50	90	32 (35)	0.45	3 (3)	0.03*	33 (36)		0.76
> 50	67	20 (30)		8 (11)		23 (34)		
Tumor size (mm)								
≤ 20	35	8 (22)	0.14	2 (5)	0.73	10 (29)		0.32
> 20	122	44 (36)		9 (7)		46 (37)		
Histological grade								
Grade I	31	15 (48)	0.005*	1 (3)	0.30	6 (19)		0.003*
Grade II	60	17 (28)		3 (5)		18 (30)		
Grade III	66	30 (45)		7 (10)		32 (48)		
Nodal involvement								
Positive	72	24 (33)	0.52	3 (4)	0.15	25 (34)		0.89
Negative	57	16 (28)		6 (10)		19 (33)		
Estrogen receptor								
Positive	60	16 (26)	0.17	2 (3)	0.15	17 (28)		0.13
Negative	97	36 (37)		9 (9)		39 (40)		
Progesterone receptor								
Positive	69	18 (26)	0.09	2 (3)	0.07	19 (27)		0.06
Negative	88	34 (38)		9 (10)		37 (42)		
Her2 status								
Positive	116	13 (11)	0.89	3 (7)	0.89	14 (35)		0.89
Negative	41	39 (97)		8 (20)		40 (100)		
EGFR								
Positive	16	4 (25)	0.46	5 (14)	0.05*	5 (31)		0.69
Negative	141	48 (34)		6 (5)		51 (36)		
Cytokeratin 5/6								
Positive	35	12 (34)	0.8	4 (11)	0.8	12 (34)		0.84
Negative	122	40 (32)		7 (6)		44 (36)		
Molecular subtype								
Luminal A	63	15(23)	0.10	2 (3)	0.33	16 (25)		0.006*
Luminal B	21	7 (33)		1 (4)		7 (33)		
HER2	20	6 (30)		2 (10)		7 (35)		
Triple negative	53	24(45)		6 (11)		26 (49)		
Triple negative subtype								
Basal-like	13	8 (61)	0.01*	5 (38)	0.001	9 (69)		0.005*
Non basal-like	40	18 (45)		2 (5)		19 (47)		

p value for Chi square or Fisher exact test

HER2 human epidermal growth factor receptor-2, *EGFR* epidermal growth factor receptor

*Significant correlations

breast cancer cases showed a stem cell phenotype with the expression of at least one of the stem cell markers. Indeed, 52 of the 157 (33%) breast cancer cases showed a strong and complete membranous CD44 expression in most tumor cells (Fig. 1). However, 10 of the 157 (7%) cases were classified as positive for ALDH1, showing a clear cytoplasmic expression in the tumor cells with a varying intensity and distribution (Fig. 2). Only 7 cases showed both CD44 and ALDH1 staining.

In the positive cases, the strong membranous expression of CD44 in the tumor cells contrasts with the absence of a detectable staining in the normal mammary epithelial cells. Furthermore, positive immunostaining for CD44 was also noted in the lymphocytes of the stroma in some cases

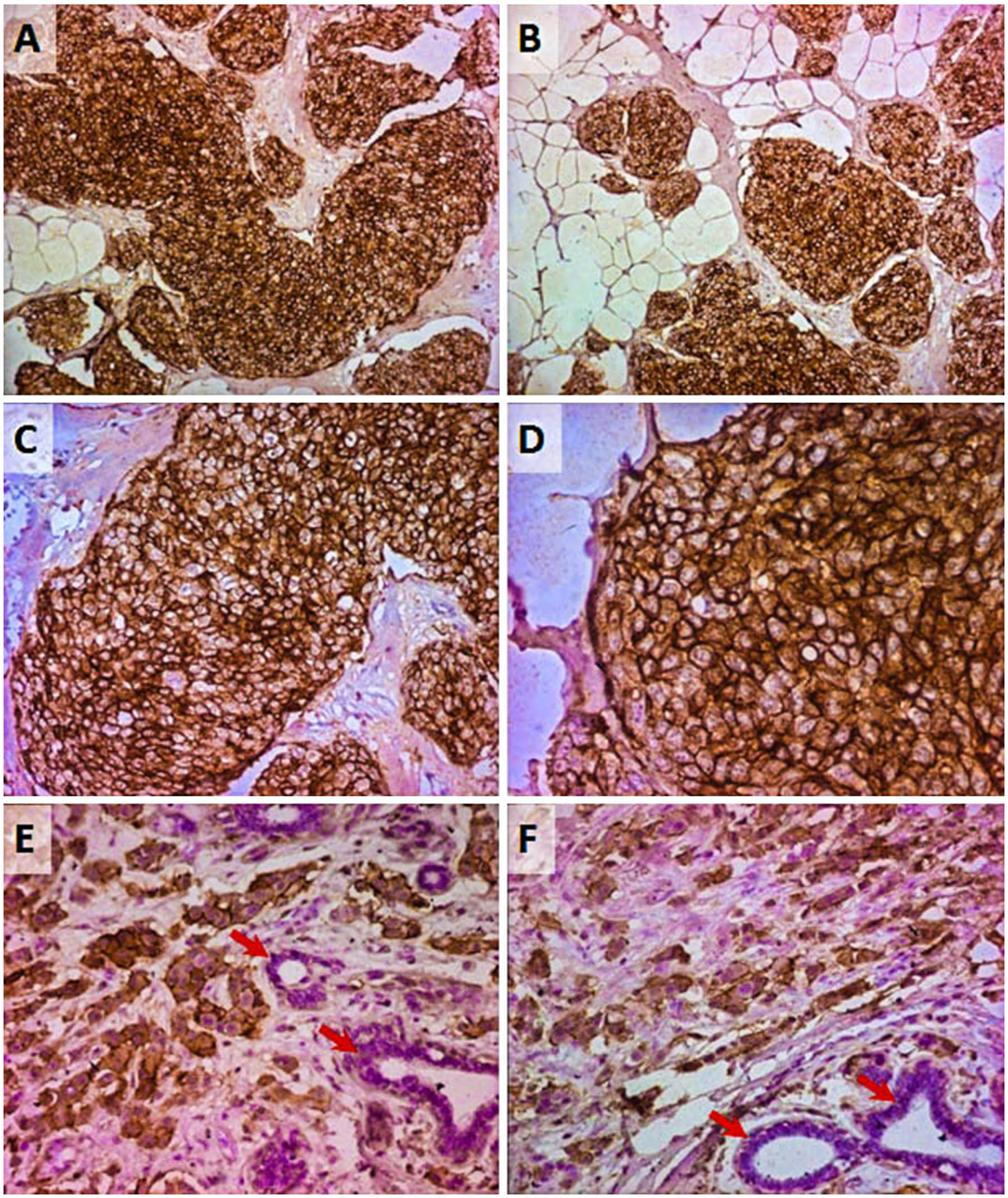

Fig. 1 Examples of immunostaining for CD44 in breast cancer (original magnification, **a, b** ×100, **c** ×200, **d–f** ×400). Strong membranous expression of CD44 in almost all of the tumor cells (brown staining), whereas the normal epithelial cells (arrows) remain negative

(Fig. 1a). Regarding ALDH1, a focal staining was observed in some normal mammary lobules (Fig. 2b).

Correlation between CD44/ALDH1 expression and clinicopathologic parameters

As shown in Table 1, we found that CD44 expression was correlated with histological grade ($p = 0.005$). For ALDH1, we found that its expression is more frequently detected in women of advanced years (> 50 years, $p = 0.03$). In addition, a strong association was found between ALDH1 expression and the basal marker EGFR ($p = 0.05$).

Considering the expression of the two markers, we showed that breast tumors with stem cell phenotype ($CD44^+$ and/or $ALDH1^+$) were significantly correlated

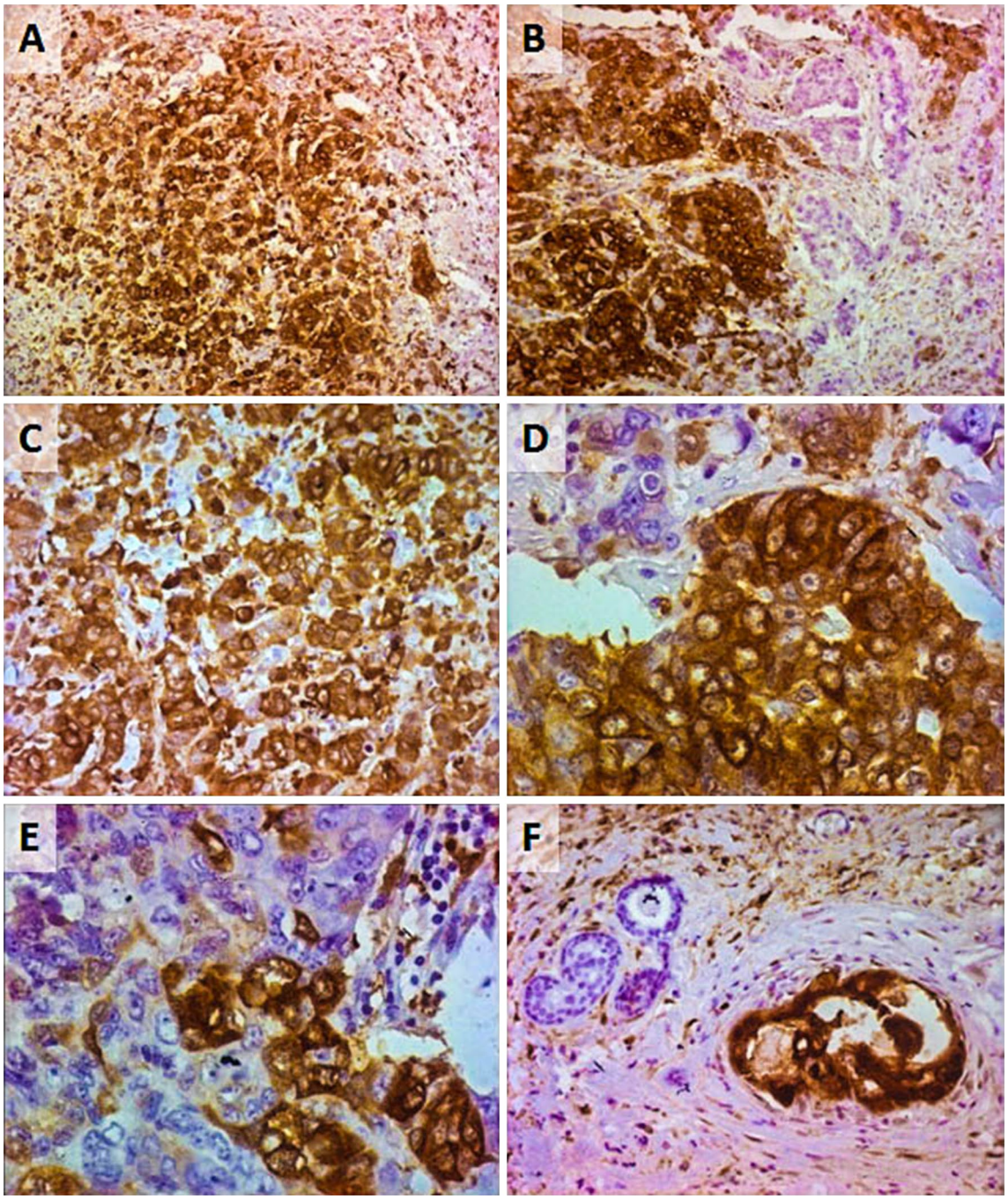

Fig. 2 Examples of immunostaining for ALDH1 in breast cancer (original magnification, **a, b** ×100, **c, f** ×200, **d, e** ×400). Note the clear cytoplasmic expression of ALDH1 in the tumor cells (brown staining), with varying intensity and distribution, whereas the normal epithelial cells (arrows) remain negative

with high histological grade, as 48% (32/66) of the positive cases were grade III ($p = 0.003$), whereas 19% and 30% of the negative cases were, respectively, grade I and grade II. Moreover, a trend for correlation with progesterone receptor negativity was found ($p = 0.06$).

Association between intrinsic molecular subtypes and the expression of breast cancer stem cell markers

Among the 157 cases investigated in this study, 63 cases (23%) were luminal A, 21 cases (33%) were luminal B, 20

cases (30%) were HER2, and 53 cases (45%) were triple negative.

Taking into account the expression of basal markers cytokeratin5/6 and EGFR, we classified triple negative tumors into two sub-groups (basal-like and non basal-like).

The investigation of the relationship between the stem cell markers and breast cancer molecular subtype (Table 1) revealed a high prevalence of stem cell phenotype in triple negative tumors (45%) compared to luminal A (23%), luminal B (33%) and HER2 (30%) ($p = 0.006$).

In triple negative group, we found that tumors with stem cell phenotype were more frequent in basal-like than non basal-like tumors ($p = 0.005$).

Discussion

In the current study, we analyzed, through a large series of breast carcinomas, the expression of stem cell markers CD44 and ALDH1 to assess whether their expression is associated with a particular clinicopathologic feature.

We found that CD44 and ALDH1 were, respectively, expressed in 33% (52 of 157) and 7% (10 of 157) of breast cancer cases. Several studies have investigated those markers in breast cancer and they have reported variable rates of their expressions ranging from 20 to 55% for CD44 [3, 15] and 5 to 35% for ALDH1 [18–26].

Several factors have been involved to explain these differences in terms of prevalence of cancer stem cell markers expression in breast cancer. Those factors include the heterogeneity of the tested series due to differences in the inclusion criteria adopted in terms of the histological types [27] and the clinical stage [24, 26, 28, 29]. In addition, those differences might be the result of the differences in the experimental protocols used and the cutoff value adopted. In our study, we adopted a cutoff value of 10% as proposed by many other studies [3, 26, 28], whereas others used 5% [19–21] and 1% [11].

With regard to the clinicopathologic parameters, we found a strong correlation between CD44 expression and high histologic grade ($p = 0.005$). The last finding suggested that increasing CD44 expression may play a role in the tumor aggressiveness. This result was in agreement with several studies showing that CD44 expression was correlated with high histological grade, tumor growth, lymph node invasion and visceral metastases [3, 24].

Previous works have shown a significant association between ALDH1 expression and clinical aggressiveness parameters such as high tumor size, high histological grade and lymph node involvement [18–26]. In our data, we found no significant association between ALDH1 expression and any of the clinicopathologic parameters investigated. This might be due to the low number of ALDH1-positive cases in our series. It seems also the same in the work of Neumeister et al. [23], who found ALDH1 positivity in only 7% of their breast cancer cases.

On the other hand, we investigated whether an association existed between cancer stem cells and the molecular subtypes of breast cancers. We found more frequent cancer stem cell phenotype (ALDH1 and/or CD44 expression) in triple negative tumors (50%) than in HER2 (36%), luminal B (33%), or luminal A (24%) groups ($p = 0.006$). This finding is in accordance with many prior reports [10, 11, 30, 31]. It is well-documented that triple negative breast carcinomas are correlated with a worse prognosis than other molecular subtypes [30].

Taking into account the expression of basal markers, we showed a strong association between the expression of breast stem cell markers and basal-like breast cancer subtype ($p = 0.005$). This finding is consistent with several previous reports showing that basal-like tumors had more cancer stem cell phenotype than the other groups [10, 31]. The presence of basal-like trait was considered as an indicator of aggressive behavior and worse prognosis [31]. It has been hypothesized that those tumors derived from mammary luminal progenitor cells (estrogen receptor negative) are blocked at an early stage of differentiation and that such blockage was in relation with early inactivation of BRCA1 gene during the carcinogenesis [32]. In fact, breast cancer developed in BRCA1 germline mutation carriers were typically of basal-like subtype, possibly due to the crucial role BRCA1 in the differentiation of estrogen receptor-negative stem cells to estrogen receptor-positive luminal cells [33]. It has been also reported that sporadic breast cancer in which BRCA1 is inactivated by promoter hypermethylation or somatic mutations show histological features and clinical outcomes similar to those found in the tumors of BRCA1 germline mutated patients [34].

Current breast cancer treatment modalities target proliferating cells, but because the breast cancer stem cells are thought to be slowly cycling cells, they may escape the treatment when not actively proliferating [35]. This last fact may explain breast cancer treatment failures and relapse. Recent knowledge has proposed that therapies targeting CD44 may destroy the cancer stem cell population [27]. Indeed, promising pre-clinical studies focusing on CD44 targeting, including a monoclonal antibody directed against this antigen, have been highlighted [27]. Currently, new humanized anti-CD44 antibodies are under preclinical investigation for anti-cancer stem cell therapy to treat patients with metastatic or locally advanced malignant solid tumors expressing CD44 [36]. Two clinical trials, including a Phase III trial in metastatic colorectal cancer, have been very disappointing [27]. The therapeutic interest in this marker in breast cancer is not yet clear. In fact, targeting CD44 might hold a great promise for the cure of

breast cancers particularly the triple negative/basal-like tumors.

Conclusions

In summary, we analyzed the expression of cancer stem cell markers CD44 and ALDH1 in a large series of breast cancer from Tunisian patients. We found that CD44 and ALDH1 are, respectively, expressed in 33 and 7% of cases. We also observed that breast tumors with stem cell phenotype were significantly correlated with the high histological grade and with the basal-like intrinsic molecular subtype. The results suggest that cancer stem cell markers especially CD44 might be interesting targets to develop new therapies particularly for triple negative/basal-like breast carcinomas.

Acknowledgements The authors acknowledge Mr. Boukataya Samir (English teacher at the Faculty of Dental Medicine, Tunisia) for English editing.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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